

GB Virus C (GBV-C) Infection in Patients With Chronic Hepatitis C. Influence on Liver Disease and on Hepatitis Virus Behaviour: Effect of Interferon Alfa Therapy

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The aim of this study was to evaluate, in patients with chronic hepatitis C, 1) the prevalence and the epidemiological characteristics of GB virus C (GBV-C) infection, 2) the influence of GBV-C on hepatitis C virus (HCV) infection, 3) the pathogenicity of GBV-C in the absence of treatment and under interferon therapy, and 4) the effect of interferon alfa on GBV-C and HCV replications. One hundred fifteen patients with chronic hepatitis C were studied. Before treatment, they were tested for GBV-C RNA by PCR and GBV-C genotype was determined for positive samples. Pre-treatment information was collected, including age, gender, source of HCV, estimated duration of HCV infection, alanine aminotransferase and gamma-glutamyl transpeptidase activities, cirrhosis and Knodell's score on liver biopsy, HCV genotype, HCV viral burden and anti-HCV core IgM antibodies. The genetic complexity of the hypervariable region 1 (HVR1) of HCV was studied by PCR-Single Strand Conformation Polymorphism. All patients were treated with 3 to 9 mega units of interferon alfa-2a three times per week for 3 to 6 months. The influence of GBV-C on the evolution of ALT and HCV replication during and after treatment was studied, and GBV-C and HCV RNA were monitored monthly by PCR during this period. Eighteen patients (16%) were GBV-C RNA-positive. Among 11 samples studied, GBV-C genotype 2a was present in 9 cases, 2b in one case and type 3 in one case. GBV-C RNA-positive patients were significantly younger than GBV-C RNA-negative ones (38.4 ± 11.5 vs. 47.4 ± 14.0 , $P = 0.012$), a result independent of the route of transmission and the disease duration. No difference between GBV-C RNA-positive and -negative patients was found for

other epidemiological parameters (e.g. gender, risk factor for parenteral viral infections, disease duration and HCV genotypes), or for the characteristics of HCV infection and related liver disease (e.g. HCV RNA level, genetic complexity of the HVR1, anti-HCV core IgM, alanine aminotransferase and gamma-glutamyl transpeptidase activities, cirrhosis and Knodell's score). GBV-C did not influence the rates of ALT normalization at months 3, 6 and 12 and of sustained hepatitis C virological response at month 12 of treatment follow-up. During treatment, GBV-C viremia became undetectable in 12 patients (67%) but relapse occurred after treatment withdrawal in all the nine patients with sufficient follow-up. In the remaining six patients (33%), GBV-C resisted interferon. Whatever the effect of interferon on GBV-C replication, the ALT levels correlated with the presence of HCV RNA. In conclusion, GBV-C infection is frequent in patients with chronic hepatitis C, who are mainly, but not exclusively, infected by GBV-C genotype 2a. GBV-C positive patients are significantly younger than GBV-C negative ones. GBV-C does not seem to affect HCV replication, liver disease and responses of HCV infection and liver disease to interferon therapy. GBV-C is sensitive to 3 mega units of interferon alfa administered three times per week in two-thirds of the patients, but

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INTRODUCTION

Since the discovery of hepatitis viruses C (HCV) and E (HEV) [Choo et al., 1989; Reyes et al., 1990] and the subsequent introduction of highly sensitive and specific immuno- and probe-based assays for the detection of antibody to these viruses or viral RNA [Mushahwar, 1991; Schlauder and Mushahwar, 1994], it is now evident that 15–20% of parenterally transmitted cases of non-A, non-B hepatitis (NANBH) and 10–15% of community-acquired hepatitis [Alter et al., 1992; Alter, 1994] are not due either to HCV or HEV infection. These cases are designated as non-A, non-B, non-C, non-E hepatitis cases (non-A-E) possibly caused by infection with an unknown virus. A recently isolated virus, belonging to the *Flaviviridae* family yet distinct from HCV, has been detected in the blood of patients with non-A-E hepatitis. This virus, GB virus C (GBV-C) [Simons et al., 1995a], and the so-called hepatitis G virus or HGV [Linnen et al., 1996], which are isolates of the same virus [Zuckerman, 1996], are closely related to GB viruses A and B [Muerhoff et al., 1995; Simons et al., 1995b]. GBV-A is apparently an animal virus that infects monkeys [Schlauder et al., 1995], as does GBV-B, although it remains unclear whether the latter can infect humans. However, it has been established that GBV-C/HGV is associated with chronic infection in man [Dawson et al., 1996; Linnen et al., 1996; Simons et al., 1995a].

The molecular characterization of the GBV-C/HGV genome showed that it is a positive-strand RNA virus belonging to a new genus within the *Flaviviridae* family, distinct from HCV [Leary et al., 1996b]. To date, the only reliable marker of infection with this virus is the detection of GBV-C/HGV RNA in body fluids and tissues by polymerase chain reaction (PCR) [Leary et al., 1996b; Linnen et al., 1996]. Little is known on the epidemiology and the pathogenicity of this newly discovered virus. Using PCR, the prevalence of GBV-C/HGV infections in blood donors from industrialized countries was found to be surprisingly high (1 to 2%) [Dawson et al., 1996]. Specific populations of patients appeared to be particularly at risk to GBV-C/HGV infection, e.g. hemophiliacs, patients with thalassemia, patients on hemodialysis, intravenous (IV) drug abusers, and patients infected by HCV and hepatitis B virus [Aikawa et al., 1996; Alter, 1996; Dawson et al., 1996; De Lamballerie et al., 1996; Hadziyannis et al., 1996; Leary et al., 1996b; Masuko et al., 1996; Simons et al., 1995a]. These results suggest transmission can occur through parenteral exposure secondary to injection of contaminated blood products, a finding recently confirmed by molecular analysis [Schmidt et al., 1996], or

IV drug abuse. In addition, sexual transmission and perinatal transmission of GBV-C/HGV are likely [Feucht et al., 1996; Pawlotsky et al., 1996b; Stark et al., 1996].

Animal studies showed that GBV-C/HGV was transmitted experimentally to non-human primates and induced persistent viremia in chimpanzees. Interestingly, liver damage could be observed only in tamarins, whereas chronically infected chimpanzees did not display any liver test or liver histology abnormality [Dawson et al., 1996; Krawczynski et al., 1996]. In man, the presence of GBV-C/HGV RNA has been reported in the serum of patients with both acute (including fulminant) and chronic liver disease [Alter HJ et al., 1997; Alter MJ et al., 1997; Dawson et al., 1996; Linnen et al., 1996; Yoshida et al., 1995]. However, it has never been possible to definitely demonstrate the role of GBV-C/HGV in liver disease in these patients, who were often coinfecting by other hepatotropic viruses. As recently stated by Alter [1996], it cannot be concluded, given the present observations, that GBV-C/HGV causes acute or chronic hepatitis. Nevertheless, whatever the real pathogenicity of GBV-C/HGV, the discovery of this new virus, possibly transmissible through blood transfusion, raises the problems of blood screening, prevention of transmission and therapeutic approaches. Especially, the effect of interferon (IFN) alfa therapy has been poorly documented.

In the present study, we used PCR primer sequences initially designed to detect the GBV-C genome in patient sera [Leary et al., 1996b]. Using this technique, we evaluated, in a large series of patients with chronic hepatitis C 1) the prevalence and the epidemiological characteristics of GBV-C infection, 2) the influence of GBV-C coinfection on the characteristics of HCV infection, 3) the pathogenicity of GBV-C in the absence of treatment as well as under IFN therapy, and 4) the effect of IFN alfa treatment on GBV-C replication, according to its effect on liver disease and HCV replication.

MATERIALS AND METHODS

Patients

One hundred fifteen consecutive patients with chronic hepatitis C (76 men, 39 women; mean age 46.0 ± 13.9 years, range 21 to 75) eligible for IFN therapy were studied between January 1992 and February 1995. The diagnosis of chronic hepatitis C was based in every case on a combination of 1) persistently elevated serum ALT activity for more than 6 months, 2) chronic active hepatitis on liver biopsy, 3) serological markers for HCV (second- or third-generation enzyme-linked immunosorbent assay (ELISA 2.0 HCV or ELISA 3.0 HCV, Ortho Diagnostic Systems, Raritan, NJ), confirmed by second- or third-generation recombinant immunoblot assay (RIBA™ 2.0 HCV strip immunoblot assay or RIBA™ 3.0 HCV strip immunoblot assay, Ortho Diagnostic Systems)), and 4) HCV RNA positivity in a highly sensitive reverse transcriptase-“nested” polymerase chain reaction (PCR) [Pawlotsky et al.,

1994]. None of the patients had markers of human immunodeficiency virus infection or hepatitis B surface antigen (HBsAg) and there was no evidence of current drug addiction, alcohol abuse or autoimmune disease. Patients under 18 years and over 75 years, patients with decompensated cirrhosis or hepatocellular carcinoma, pregnant women, and patients with depression were excluded. Exclusion criteria also included albuminemia lower than 30 g/L, prothrombin time lower than 50% of the control, bilirubin higher than 60 μ mol/L, platelet count lower than 70,000 G/L, neutrophil count lower than 1500 G/L, hemoglobin lower than 11 g/dL and serum creatinine higher than 120 μ mol/L. None of the patients had record of previous antiviral or immunomodulatory treatment. The trial was approved by the institutional ethics committee and all the patients gave their written informed consent.

Study Protocol

Before treatment, the 115 patients were tested for GBV-C infection by reverse transcription-PCR. GBV-C genotype was determined for GBV-C RNA-positive samples. The following pretreatment information was collected: age, gender, apparent source of HCV infection (blood transfusion, IV drug abuse or unknown source), estimated duration of HCV infection in patients with an identified risk factor, serum alanine aminotransferase (ALT) and gamma-glutamyl transpeptidase (γ -GT) activities, presence of cirrhosis on liver biopsy, HCV genotype, serum level of HCV RNA (viral burden) and serum anti-HCV core IgM antibodies. The genetic complexity of the hypervariable region 1 (HVR1) of the HCV genome was also studied before treatment by means of a PCR-Single Strand Conformation Polymorphism (SSCP) technique [Orita et al., 1989] developed in our laboratory. GBV-C RNA-positive and -negative patients were compared for all of these pretreatment parameters.

All 115 patients were treated with 3 mega units of IFN alfa-2a (RoferonTM-A, Roche Laboratories, Basel, Switzerland) subcutaneously three times a week for at least 3 months. Serum ALT activity was used as a biochemical index of the response to IFN. At month 3 of therapy, patients with ALT remaining higher than the upper normal limit were considered nonresponders to the standard dosage of IFN used in the study. They were randomized into three groups: one group withdrew therapy, one group received a further 3 months of treatment at the same dose, and one group received 9 mega units of IFN thrice a week for 3 additional months. Patients with normal ALT activity at month 3 (initial responders) all received a further 3 months of treatment at the same dose. Serum ALT activity was measured at month 6, i.e. when IFN was discontinued in all patients (biochemical response upon treatment withdrawal), and at month 12, i.e. at least 6 months after treatment withdrawal. At the latter date, a sustained biochemical response to the standard dosage of IFN was defined as normal ALT activity. All patients with a sustained biochemical response at month 12 un-

derwent HCV RNA determination by non-quantitative PCR. Patients who were PCR-negative at this time were defined as sustained virological responders. The influence of GBV-C infection on the ALT normalization at month 3, the ALT normalization at month 6 (treatment withdrawal) and the sustained virological response of hepatitis C to therapy (HCV RNA clearance at month 12) was studied.

In addition, in all of the patients with GBV-C RNA detected before treatment, GBV-C RNA and HCV RNA were monitored monthly by non-quantitative PCR during IFN therapy and the posttreatment follow-up period until month 12.

Detection of GBV-C RNA by PCR

Briefly, RNA was extracted from 50 μ l of serum with RNAzol (RNA-B, Bioprobe Systems, Montreuil-sous-Bois, France) and chloroform, and reverse transcribed at 42°C for 90 min by using 100 pmol of random hexamers in the presence of 8 units of avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI). Amplification was performed by "touch-down" PCR using 1 μ mol each downstream consensus degenerated primer gbvc-a1 (5'-ACNACNAGGTCNC-CRTCYTTGATGAT-3') and upstream primer gbvc-s1 (5'-GGNRMKRTYCCYTTTATGGGCATGG-3') [Leary et al., 1996c] with 5 units of *Taq* DNA polymerase (Pharmacia Biotech, Uppsala, Sweden) for 43 cycles (94°C, 20 sec; 55°C, 30 sec decreasing by 0.3°C per cycle; 72°C, 1 min). This was followed by 10 cycles of amplification (94°C, 20 sec; 40°C, 30 sec; 72°C, 60 sec) with a final 10 min extension. Positive and negative controls were included in each series and the pretreatment samples were tested in duplicate. Amplified products were analyzed by electrophoresis through 3% NuSieve agarose gel (FMC, Rockland, MD) and staining with ethidium bromide. A positive result was defined by a visible DNA fragment of 188 bp and confirmed by hybridization to a specific oligonucleotide probe (5'-GCCGGCCAGTTCTCHGCNMGGGGGGT-NAATGCTATYGCCTATTA-3') by means of the Gen. Eti.KTM DEIA kit (Sorin Biomedica, Italy) [Fiordalisi et al., 1996].

Determination of GBV-C Genotypes

GBV-C RNA-positive specimens, determined by RT-PCR as described above, were also tested using a single-tube, automated, probe-based RT-PCR assay that uses primers derived from the 5'-untranslated region (UTR) of GBV-C [Leary et al., 1996a; Muerhoff et al., 1996a]. The automated assay was carried out using RNA extracted from 25 μ l of serum using the QIAmp HCV kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. RNA was eluted from the column in 100 μ l of DEPC-treated water and one-fifth was used in the assay. RT-PCR is performed using adamantane-labeled sense and antisense primers and *rTth* DNA polymerase to perform cDNA synthesis and amplification reactions. The amplified product is then hybridized to a carbazole-labeled oligonucleotide probe

complementary to a sequence internal to the region amplified. Detection is performed using a Microparticle Enzyme Immunoassay [Fiore et al., 1988] where the probe-amplicon hybrid is sequestered on anti-carbazole coated microparticles followed by detection of the amplicon by using alkaline phosphatase-conjugated anti-adamantane antibody and 4-methylumbelliferyl phosphate as the substrate. The rate of light emission produced upon dephosphorylation of the substrate is reported as counts per second per second by the analyzer. Samples which returned rates greater than 100 (data not shown) were considered positive for GBV-C RNA [Leary et al., 1996a].

Samples determined to be positive for GBV-C RNA by the automated assay were used in RT-PCR experiments to amplify a major portion of the GBV-C 5'-UTR. Phylogenetic analysis of the resulting 5'-UTR sequences was performed as previously described [Muerhoff et al., 1996b] in order to determine the GBV-C genotype. Briefly, 7.5 μ l of the RNA obtained from the Qiagen column (see above) was reverse-transcribed using the RNA PCR kit (Perkin Elmer, Foster City, CA) in the presence of random hexamers. One-fifth of the cDNA product was amplified with 0.5 μ M primers ntrC-S1 (5'-CACTGGGTGCAAGCCCCAGAA-3') and ntrC-5R (AGCAGAAGGACTGCCCATGAT-3'), located within the 5'-UTR of GBV-C, to produce a product of approximately 540 nucleotides [Muerhoff et al., 1996a]. Thermocycling conditions were as described previously [Muerhoff et al., 1996a]. PCR products were separated by electrophoresis through a 2% agarose gel and then excised and purified using the QIAEX Gel Extraction kit (Qiagen). Purified PCR products were sequenced directly on an ABI Model 373 DNA sequencer using the ABI Sequencing Ready Reaction kit (Perkin Elmer) and gene-specific primers.

Nucleotide sequences were aligned using PILEUP (Wisconsin Sequence Analysis Package, GCG, Madison, WI). Included in the alignment and subsequent phylogenetic analysis were GBV-C 5'-UTR sequences obtained from globally distributed individuals for which the GBV-C genotype had been determined [Muerhoff et al., 1996b]. Evolutionary distances between sequences were calculated by the DNADIST program of the PHYLIP package, version 3.5c [Felsenstein, 1989]. The computed distances were used for the construction of a phylogenetic tree using the least-squares method of the program FITCH. The robustness of the resulting tree was assessed by bootstrap resampling (100 data sets) of the multiple sequence alignment using the programs SEQBOOT, DNADIST, and NEIGHBOR. The consensus tree was calculated by CONSENSE. The final output was generated using DRAWTREE.

Evaluation of the Genetic Complexity of the HVR1 Region of the HCV Genome

A standardized and reproducible PCR-SSCP method was developed in our laboratory. RNA was extracted from 50 μ l of serum with RNAzol and chloroform, and

reverse transcribed at 42°C for 90 min by using 7 pmol of the downstream primer (5'-GGTGTGGAGGGAGT-CATTGCAGTT-3', nucleotide position 1611-1634) [Enomoto et al., 1994] in the presence of 8 units of AMV reverse transcriptase. PCR was performed using 5 pmol each downstream primer and upstream primer (5'-GCTTGGGATATGATGATGAACTGGTC-3', nucleotide position 1284-1309) [Enomoto et al., 1994] with 2.5 units of *Taq* DNA polymerase. After a 5-min denaturation at 94°C, PCR was performed with 45 cycles (94°C, 1 min; 68°C, 1 min; 72°C, 1 min). Amplified products were analyzed by electrophoresis through 3% NuSieve agarose gel and staining with ethidium bromide.

The amplified products were extracted from the agarose gel and purified with the Sephaglas BandPrep Kit (Pharmacia Biotech) according to the supplier's instructions. Purified PCR products were eluted in 20 μ l of distilled water. SSCP analysis was performed by using the PCR Fragment Analysis Kit and the Multiphor II electrophoresis chamber (Pharmacia Biotech). Approximately 50 ng of amplified DNA recovered from each serum sample was diluted in 4.5 μ l of sterile distilled water, and 4.5 μ l of a solution containing 10 mM NaOH, 2 mM EDTA and bromophenol blue was added. The samples were denatured for 10 min at 100°C and quickly chilled on ice. Eight microliters of the denatured samples was loaded into the wells of a washed and dried discontinuous polyacrylamide gel (CleanGel, Pharmacia Biotech) which had been rehydrated to a 0.5 mm thick gel. Horizontal electrophoresis was run in the Multiphor II electrophoresis apparatus at a temperature of 9°C by means of an anodal and a cathodal buffer strips in contact with the two electrodes (100 V, 20 min; 600 V, 60 min).

The gel was then submitted to a fast and sensitive silver staining procedure using the DNA Silver Staining Kit (Pharmacia Biotech), a method that allows detection of 0.5 to 2 ng of DNA. For this, the gel was fixed for 30 min at room temperature in a solution containing 10% acetic acid, washed three times and incubated for 30 min in 200 ml of a solution containing 0.1% AgNO₃ (w/v) and 0.1% formaldehyde. The gel was rinsed, placed into 200 ml of a solution containing 2.5% Na₂CO₃, 0.1% formaldehyde and 0.002% sodium-thiosulfate, and slowly agitated until staining became visible. The reaction was stopped by a 20-min incubation in 10% acetic acid and staining was then preserved by a 20-min incubation at room temperature in 5% glycerin and 10% acetic acid.

After scanning of the gels, the band patterns were analyzed by means of the ImageMaster 1D software (Pharmacia Biotech). The optical density of each band was determined in each lane and those with an optical density higher than 0.03 were analyzed. The number of bands detected in each lane reflected the genetic complexity of the major variant of each HCV strain in the HVR1 region. This was confirmed by cloning and sequencing the amplified products from three samples of this study (data not shown).

Other Parameters of HCV Infection

HCV genotype was determined by using the Inno-LiPA HCV method (Innogenetics S.A., Gent, Belgium) [Stuyver et al., 1993]. The highly conserved 5'-UTR of the HCV genome was amplified by a reverse-transcriptase "nested" PCR using two sets of universal HCV primers. The amplified products were then hybridized to immobilized oligonucleotide probes specific for the different HCV genotypes as described recently [Pawlotsky et al., 1995b; Stuyver et al., 1993].

HCV RNA was quantified in 50 μ l of serum by means of the recently developed second-generation signal amplification assay based on branched DNA (bDNA) technology (Quantiplex™ HCV RNA 2.0, Chiron Diagnostics, Emeryville, CA), according to the manufacturer's instructions [Detmer et al., 1996; Kolberg et al., 1994]. In contrast to the initial assay, this version quantifies HCV RNA independently of the HCV genotype [Detmer et al., 1996; Pawlotsky et al., 1996a]. The mean value of duplicate determinations, performed independently on each serum sample, was compared to a standard curve constructed in each run from standards containing known amounts of synthetic HCV RNA. The results were expressed in millions of genome equivalents per ml or megaequivalents per ml (MEq/ml). The manufacturer's stated cutoff of the assay is 0.2 MEq/ml.

HCV RNA was tested for in the serum of patients with sustained biochemical responses to IFN therapy at month 12, and monthly from month 0 to month 12 in the patients with a GBV-C/HCV coinfection by using a recently described reverse transcription "nested" PCR procedure [Pawlotsky et al., 1994]. This procedure has been evaluated and validated for both sensitivity and specificity in a recent multicenter quality control trial [French Study Group for the Standardization of Hepatitis C Virus Polymerase Chain Reaction, 1994].

Finally, the HCV IgM EIA kit (Abbott Diagnostica, Wiesbaden, Germany) was used for detection of anti-HCV core IgM, according to the manufacturer's instructions as previously described [Pawlotsky et al., 1995a].

Statistical Analysis

The results are presented as means \pm 1 standard deviation or as percentages. The non-parametric Mann and Whitney test was used to test quantitative variables; the Chi square test and, when necessary, Fisher's exact test, were used to test categorical variables. All analyses were performed using BMDP statistical software (BMDP Statistical Software Inc., Los Angeles, California).

RESULTS

Prevalence of GBV-C Infection and GBV-C Genotypes

Among the 115 patients with chronic hepatitis C, 18 (16%) were found to have GBV-C RNA in their serum by RT-PCR assay using the NS3-derived GBV-C prim-

ers. Serum was available for determination of GBV-C genotype in 13 of them. Two of the sera tested positive for GBV-C RNA by the automated assay which utilized primers derived from conserved regions of the 5'-UTR but did not produce a PCR product when the ntrC-S1 and 5R primers were used to generate a product for subsequent sequencing. Sequences were obtained from the remaining 11 patients. Alignment of these sequences with those reported earlier [Muerhoff et al., 1996b] followed by phylogenetic analysis produced the unrooted tree shown in Figure 1. Analysis of the GBV-C 5'-UTR sequences reported here along with the original 42 isolates and the homologous region from the two HGV isolates [Linnen et al., 1996] confirmed our previous results [Muerhoff et al., 1996b]. No evidence for the existence of a GBV-C genotype unique to the dually infected chronic hepatitis patients from the present study was found. GBV-C genotype 2a was present in nine patients, 2b in one and type 3 in one case.

Epidemiological Parameters in GBV-C Infection

GBV-C RNA positive patients were 11 men and 7 women. The gender distribution was not different from that in HCV-positive, GBV-C RNA-negative patients (Table I). Their mean age was 38.4 ± 11.5 years and was significantly lower than that of GBV-C RNA-negative patients (47.4 ± 14.0 , $P = 0.012$, Table I). Among the 18 GBV-C RNA-positive patients, eight (44%) had been transfused, six (33%) were former IV drug abusers and four (22%) had no risk factor for parenteral viral infections. The prevalence of GBV-C infections tended to be higher among IV drug abusers than in transfused patients or patients with an unknown cause of infection (23% vs. 16% and 10%, respectively), but the difference was not significant. The putative duration of the disease in transfused patients and IV drug abusers did not differ between GBV-C RNA-positive and -negative patients. To determine whether the younger age of GBV-C RNA-positive patients could be related to more frequent history of IV drug abuse, we compared the ages of GBV-C-positive and -negative patients in each risk group. Among patients who received blood transfusion, GBV-C RNA-positive subjects were significantly younger than GBV-C RNA-negative ones (41.5 ± 11.2 vs. 51.6 ± 13.0 years, $P = 0.05$). GBV-C RNA-positive patients also tended to be younger than GBV-C RNA-negative ones in IV drug abusers and patients with no risk factors for parenteral viral infections (29.5 ± 2.9 vs. 32.4 ± 5.4 and 45.7 ± 13.6 vs. 50.8 ± 12.8 , respectively), but the differences did not reach significance.

The distribution of HCV genotypes in the study population is presented in Table I according to the GBV-C RNA status of the patients. The prevalence of GBV-C infection tended to be higher in the patients infected by HCV genotypes 1a (24%) and 3a (27%) than in the patients infected by HCV genotypes 1b (12%), 2 and 4 (5%), but the relationship was not significant ($P = 0.13$).

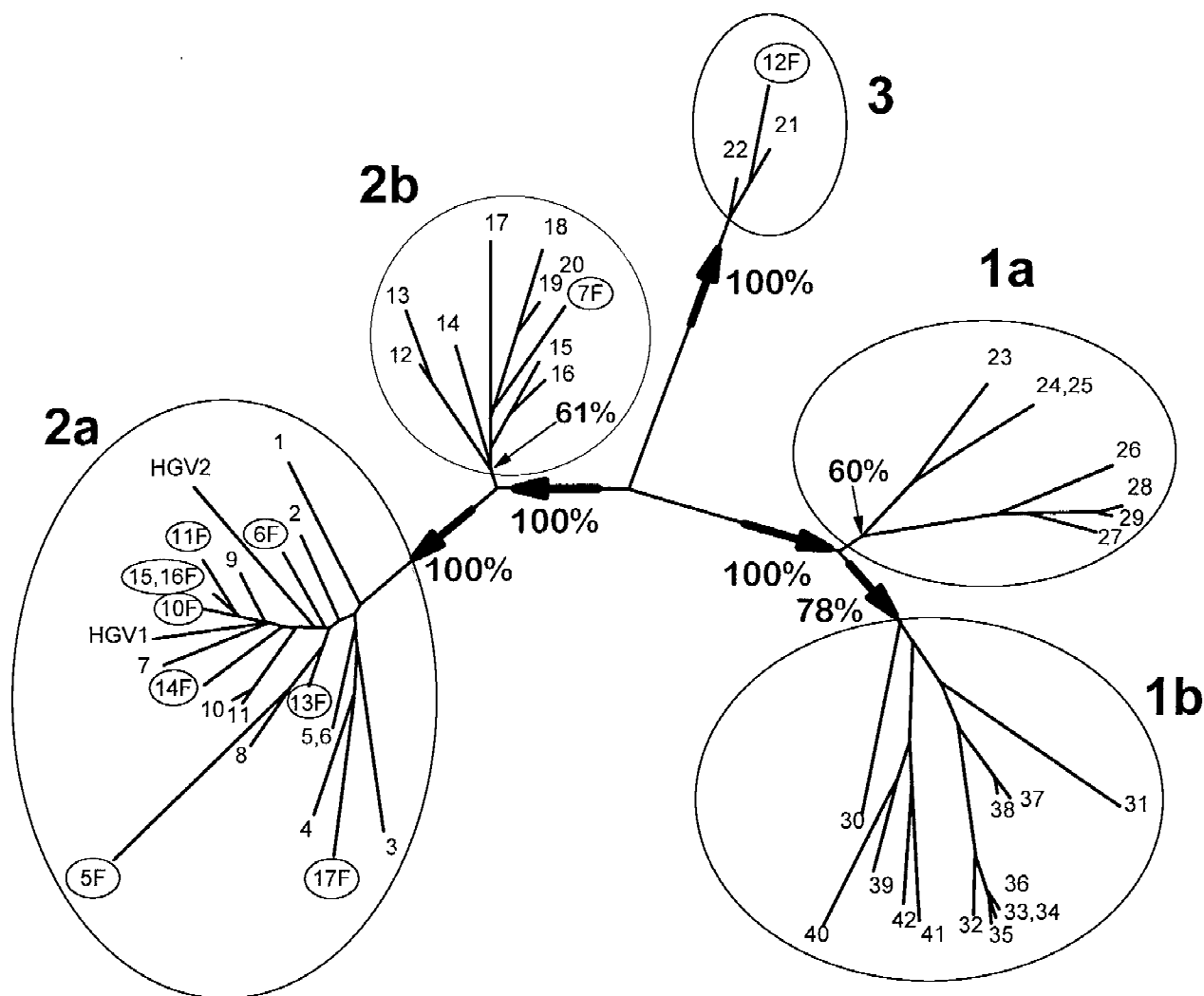


Fig. 1. Phylogenetic relationships between GBV-C isolates based upon comparison of the 5'-untranslated region. The program FITCH was used to construct the unrooted tree from the pairwise evolutionary distances calculated by DNADIST (the branch lengths are proportional to the calculated distances). Multiple sequence alignments generated using PILEUP were randomly resampled using SEQBOOT. The numbers next to bold arrows indicate the frequency with which

the group ahead of the arrow occurred out of 100 resamplings of the data. The five GBV-C genotypes (1a, 1b, 2a, 2b, 3) are grouped by the large circles. The isolates examined in this study are circled and indicated by the "F" following the patient number. HGV1 and HGV2 refer to the corresponding 5'-UTR sequences from isolates PNF2161 and R10291, respectively, reported by Linnen et al. (1996).

Influence of GBV-C Infection on HCV Infection

Table I shows the various parameters of HCV infection according to the presence of GBV-C RNA in serum. None of the pretreatment indicators of liver disease, including ALT and γ -GT levels, the presence of cirrhosis and the Knodell's score on liver biopsy, were found to differ between GBV-C RNA-positive and -negative patients. Neither the HCV RNA level, nor the genetic complexity of HCV differed between the two groups. Finally, the prevalence of anti-HCV core IgM was similar in GBV-C RNA-positive and negative patients.

Influence of GBV-C Infection on the Responses to IFN Alfa Therapy

The rates of biochemical responses (ALT normalization) at months 3, 6 and 12 and of sustained hepatitis

C virological response (ALT normalization and absence of PCR-detectable HCV RNA at month 12) to 3 mega-units three times per week of IFN alfa-2a were 50/115 (43%), 34/112 (30%), 11/112 (10%) and 8/112 (7%), respectively, in the patients with chronic hepatitis C. Table II shows these response rates according to the presence of GBV-C RNA in serum. No significant difference in response rate was found between GBV-C RNA-positive and -negative patients.

Effect of IFN Alfa on GBV-C and HCV Replications in Patients with Mixed Infections

Figure 2 shows key examples of the effect of IFN alfa on ALT activity, and GBV-C and HCV replication according to the dose and duration of treatment in patients with HCV and GBV-C coinfection. As shown in

TABLE I. Epidemiological Parameters and Characteristics of HCV Infection and Related Liver Disease According to the Detection in Serum of GBV-C RNA in 115 Patients With Chronic Hepatitis C

	All patients (n = 115)	GBV-C RNA- negative (n = 97)	<i>P</i>	GBV-C RNA- positive (n = 18)
Male sex	76 (66%)	65 (67%)	NS	11 (61%)
Age	46.0 ± 13.9	47.4 ± 14.0	0.012	38.4 ± 11.5
Route of transmission			NS	
blood transfusion	49 (43%)	41 (42%)		8 (44%)
IV drug abuse	26 (23%)	20 (21%)		6 (33%)
unknown	40 (35%)	36 (37%)		4 (22%)
Disease duration (months) ^a	149.1 ± 101.2	152.3 ± 101.5	NS	134.3 ± 102.1
HCV genotype			NS	
1a	21 (18%)	16 (17%)		5 (28%)
1b	51 (44%)	45 (46%)		6 (33%)
3a	22 (19%)	16 (17%)		6 (33%)
others	21 (18%)	20 (21%)		1 (6%)
ALT (N < 45 IU/L)	170.4 ± 123.1	175.1 ± 127.9	NS	145.5 ± 92.2
γ-GT (N < 65 IU/L)	83.6 ± 95.8	75.8 ± 80.6	NS	128.2 ± 152.5
Cirrhosis	23 (20%)	20 (21%)	NS	3 (17%)
Knodell's score	8.9 ± 3.1	8.8 ± 2.9	NS	9.5 ± 3.5
HCV RNA (MEq/ml)	5.4 ± 6.6	4.9 ± 5.9	NS	7.8 ± 9.6
Genetic complexity (bands)	5.3 ± 2.0	5.3 ± 2.1	NS	5.3 ± 1.9
Anti-HCV core IgM	49 (43%)	42 (43%)	NS	7 (39%)

^aOnly in transfused patients and IV drug abusers. NS: Not significant.

TABLE II. Influence of GBV-C Infection on ALT Normalization and Sustained Clearance of HCV RNA in 115 Patients With Chronic Hepatitis C Treated With Interferon Alfa-2a

	GBV-C RNA- negative (n = 97)	<i>P</i>	GBV-C RNA- positive (n = 18)
Response to IFN alfa			
Normal ALT at month 3	44/97 (45%)	NS	6/18 (33%)
Normal ALT at month 6	30/96 (31%)	NS	4/16 (25%)
Normal ALT at month 12	10/96 (10%)	NS	1/16 (6%)
Sustained HCV RNA clearance	7/96 (7%)	NS	1/16 (6%)

IFN, interferon; ALT, alanine aminotransferase activity; NS, not significant.

the figure, the responses of GBV-C and HCV to IFN treatment were independent.

In six of the 18 patients (33%), GBV-C resisted IFN treatment and GBV-C RNA remained detectable during the entire follow-up period. GBV-C viremia became undetectable in the remaining 12 patients (67%) during IFN treatment. The disappearance of GBV-C RNA from serum occurred within the first month of treatment in seven patients, at month 2 in three patients and at month 3 in one patient. In one case (patient 12 in Figure 2), GBV-C RNA was cleared from serum only when the dosage of IFN was increased from 3 to 9 megaunits three times per week. Among the 12 patients who initially responded to IFN, three were lost

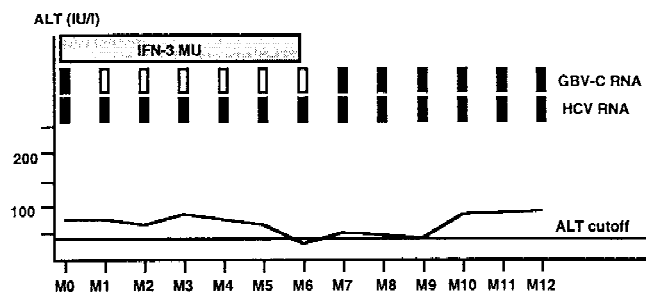
for follow-up or withdrew therapy due to severe side-effects and nine had a prolonged follow-up after IFN withdrawal. In these nine patients, the recurrence of GBV-C infection was constant after IFN withdrawal and occurred within 1 or 2 months in almost all cases.

Whatever the effect of IFN treatment on GBV-C replication, the ALT levels correlated with the presence of HCV RNA, suggesting that GBV-C replication did not play a role in liver enzyme perturbations. Indeed, ALT remained elevated in four patients who became negative for GBV-C RNA without clearance of HCV RNA. In contrast, in two patients, GBV-C recurrence in the absence of HCV recurrence was associated with persistently normal ALT. In two patients, however, ALT ac-

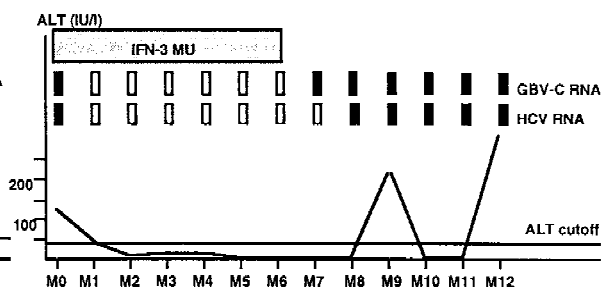
Fig. 2. Key examples of the evolutions of the alanine aminotransferase (ALT) activity, of GBV-C replication and of HCV replication assessed by PCR according to the dose and duration of the interferon (IFN) treatment in patients with HCV and GBV-C coinfection. Evolution of ALT is indicated by the black line. Black boxes mean positive PCR and white boxes mean negative PCR. Duration and dose of IFN are indicated in the grey box. The responses of GBV-C and HCV to IFN treatment were independent. GBV-C viremia became undetectable in patients 2, 3, 4, 9 and 10 with 3 units mega three times per

week of IFN alfa-2a, and in patient 12 when the dosage of IFN was increased to 9 mega units, but the recurrence of GBV-C infection was constant after IFN withdrawal. In patients 15 and 16, GBV-C resisted IFN treatment. Whatever the effect of IFN treatment on GBV-C replication, the ALT levels correlated with the presence of HCV RNA. For instance, ALT remained elevated in patients 2 and 10, who became negative for GBV-C RNA without clearance of HCV RNA, while GBV-C recurrence in the absence of HCV recurrence was associated with persistently normal ALT in patients 4 and 12.

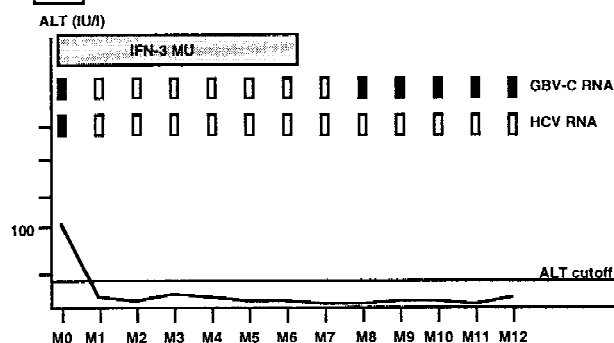
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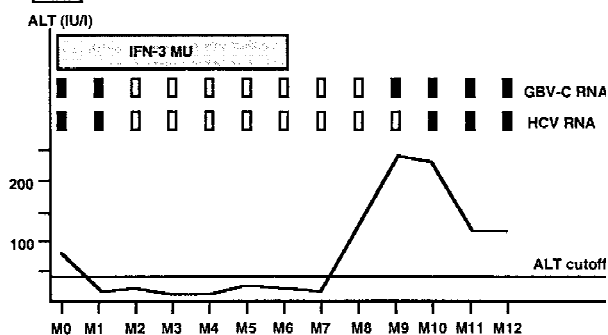
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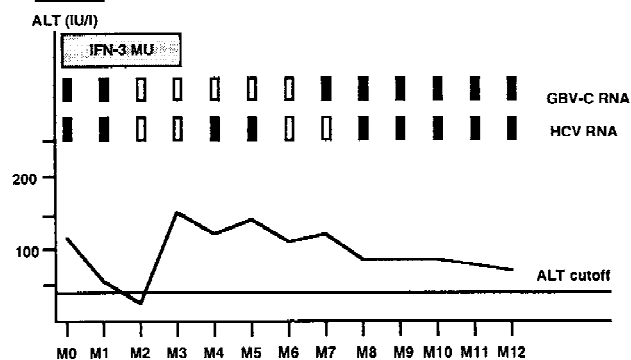
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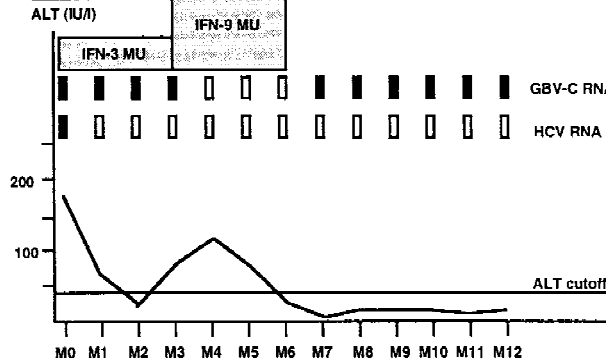
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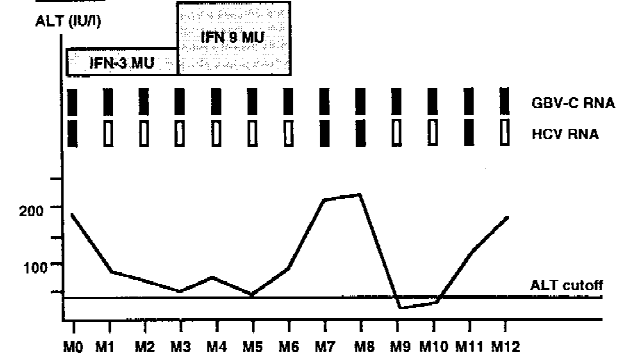
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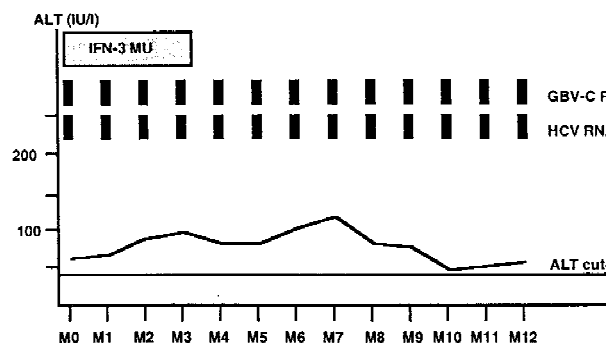
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#16



tivity remained elevated throughout the entire follow-up period, despite transient loss of detection of both GBV-C and HCV.

DISCUSSION

This study confirms the high prevalence of GBV-C/HGV infections (16%) reported previously in patients infected by HCV in industrialized countries [Dawson et al., 1996; Hadziyannis et al., 1996; Linnen et al., 1996] and provides new insights into the epidemiology of this recently discovered virus. Previous analysis of 42 GBV-C 5'-UTR sequences obtained from 35 globally distributed individuals provided evidence for the existence of GBV-C genotypes [Muerhoff et al., 1996b]. The major GBV-C genotypes demonstrated a strong correlation with geographic origin in that type 1, type 2, and type 3 isolates were found exclusively in Western Africa (Ghana), the United States and Europe, or Asia (Japan), respectively. Inclusion of the sequences from the 11 isolates reported here in an analysis of all available 5'-UTR sequences provided no evidence for new types or subtypes of GBV-C in France or for a subtype associated with dual HCV/GBV-C infection among chronic hepatitis patients. Bootstrap analysis of 100 random resamplings of the alignment data supported the existence of the five previously identified genotypes. Interestingly, 10 of the 11 patients were infected with GBV-C genotype 2 (subtype 2a in nine cases and subtype 2b in one case). The remaining patient, who had no apparent risk factor for parenteral viral infections, was infected with GBV-C genotype 3, a subtype previously defined by only two sequences and only found in Asia. It is interesting to note that this patient travelled to Iran and Sahara in the 1960s, although it remains unknown whether GBV-C genotype 3 was present in these countries at this time. While a correlation between GBV-C genotype 2a and co-infection with HCV in these patients is apparent, genotype 2a is the most common subtype observed in Europe to date and a complete epidemiological survey of GBV-C subtypes has not been undertaken. Thus, it would be premature to attribute unique biological properties to any of the GBV-C subtypes at present.

It is interesting to note that the patients infected by GBV-C were significantly younger than the patients without GBV-C infection, while we recently observed similar findings in a series of 61 HCV-infected pregnant women [Pawlotsky et al., 1996b]. This was especially the case in the subgroup of patients with a history of blood transfusion, thus eliminating a confounding role of IV drug abuse in the relationship between young age and GBV-C infection. However, no relationship was found between the presence of GBV-C infection and the duration of the disease estimated by the date of transfusion. This suggests that, although these patients were exposed to the risk of parenteral transmission (but they were selected on the presence of chronic HCV infection), at least some of them might have been infected through another route, especially in the group of transfused patients. In this respect,

mother-to-infant transmission of GBV-C/HGV was recently observed and the role of sexual or person-to-person transmission of this virus needs to be further investigated [Feucht et al., 1996; Pawlotsky et al., 1996b; Stark et al., 1996]. In addition, GBV-C might have been introduced in Europe more recently than other viruses such as HCV. Finally, it is possible that GBV-C replication can resolve spontaneously after some years of infection [Tacke et al., 1977], thus explaining that only younger patients, likely to have been infected more recently, were found to be viremic. The latter hypotheses are both consistent with the recent observation of a higher GBV-C/HGV RNA prevalence among persons who had been injecting drugs for less than 5 years than in subjects with longer IV drug use [Stark et al., 1996].

GBV-C infection appeared to be more frequent in patients infected by HCV genotypes 1a and 3a than in patients infected by other HCV genotypes. This tendency was likely due to the significant association of these genotypes with IV drug abuse in industrialized countries we recently reported [Pawlotsky et al., 1995b], and to the tendency towards a higher prevalence of GBV-C infection in drug abusers. There was no argument in our study to support the hypothesis that GBV-C infection could be preferentially associated with peculiar HCV genotypes for virological rather than epidemiological reasons.

We showed, in the present study, the lack of any influence of GBV-C infection on the virological characteristics of HCV infection (Table 1), making unlikely a viral interaction in vivo between these two viruses. In particular, GBV-C did not appear to alter the level of HCV replication determined prior to IFN treatment, in contrast with other viruses, such as hepatitis B and D viruses, where coinfection with these viruses is associated with significantly lower HCV RNA levels [Crespo et al., 1995; Liaw, 1995; Zarski et al., 1995]. GBV-C was shown to belong to the same family as HCV, namely the *Flaviviridae*, and sequence analysis of several GBV-C strains revealed that the gene encoding the viral capsid was lacking or truncated in a significant proportion of cases [Desai et al., 1996; Erker et al., 1996; Muerhoff et al., 1996b; Simons et al., 1996]. To explain the frequency of GBV-C infections in HCV-infected patients, it could be hypothesized that GBV-C would use HCV capsids to replicate and, thus, compete with HCV to survive. However, several arguments make this hypothesis unlikely. They include 1) the lack of influence of GBV-C infection on HCV replication in this study, 2) the independent responses of HCV and GBV-C to IFN alfa therapy, 3) our recent finding that mother-to-infant transmission of GBV-C is possible, but is independent of HCV transmission [Pawlotsky et al., 1996b], and 4) the recent findings of Simons et al. [1996] that demonstrate GBV-C polyprotein translation initiation occurs immediately upstream of the E1 gene. The mechanism by which GBV-C can be encapsidated remains to be elucidated.

In the present study, no relationship was found be-

tween the presence of GBV-C and the genetic complexity of HCV strains as assessed by PCR-SSCP analysis. The technology we developed in our laboratory allows quantification of the number of different HVR1 sequences present in a given patient at a given time during infection as a result of the "quasispecies" distribution of HCV [Martell et al., 1992]. This technique allows the detection of sequences that represent more than 10% of the entire viral population (data not shown), i.e. it allows the examination of the genetic complexity of the major variants of HCV. The quasispecies distribution of HCV is the result of an interaction between 1) HCV replication, with frequent spontaneous mutations due to the lack of "proofreading" activity of the HCV RNA-dependent RNA polymerase, and 2) the selection of escape mutants by the immune response of the host (responsible for the synthesis of neutralizing antibodies directed to the envelope epitopes encoded by the HVR1 region of the genome) that ultimately develop and become dominant [Martell et al., 1992; Weiner et al., 1992]. The lack of influence of GBV-C infection on HCV genetic complexity is consistent with the apparent lack of influence of this virus on HCV replication and on the neutralizing response directed to HCV. Since GBV-C is an RNA virus belonging to the same family as HCV and possibly responsible for the establishment of chronic infections, it is highly probable that this virus also exists as a quasispecies in infected individuals, the role of which in the pathogenesis of infection remains to be studied.

The main question raised by the recent discovery of GBV-C/HGV is whether this virus is responsible for a disease and, if so, whether this disease involves the liver. This point is of major importance because, if GBV-C is actually pathogenic, diagnostic tests must be developed, blood screening must be implemented with these tests and infected patients must be diagnosed and treated with effective drugs. Although our study does not definitely answer the question of the pathogenicity of GBV-C alone, it confirms previous results [Tanaka et al., 1996] by showing that GBV-C does not play a role in liver disease when associated with HCV. This is in contrast with hepatitis B or D viruses, which have been shown to aggravate HCV-induced disease when present [Crespo et al., 1995; Liaw, 1995]. In our study, neither the biochemical (ALT and γ -GT) nor the histological (cirrhosis and Knodell's score) parameters of liver disease differed between GBV-C RNA-positive and -negative patients. The responses to IFN therapy at different times of follow-up [Pawlotsky et al., 1996c] of both liver disease (assessed by ALT levels) and HCV infection (assessed by HCV RNA PCR) were not affected by the presence of GBV-C coinfection. In addition, we observed that, during IFN therapy, as well as after IFN withdrawal, the ALT activity correlated with HCV replication, but not with GBV-C replication. In two patients, however, persistent elevation of ALT was explained neither by HCV nor by GBV-C replication. One can hypothesize that, in these two patients, HCV was still replicating at levels lower than the cutoff of

the PCR assay, because both experienced HCV relapse a few months later.

Finally, we demonstrated in this study that, in patients coinfecting by HCV, GBV-C (genotype 2a in most cases) is sensitive to a standard dose of IFN alfa in two-thirds of the cases and that the response is early in most instances, whereas it is resistant in the remaining cases. However, we also showed that, in the patients who experience a GBV-C response to IFN, relapse is constant and usually occurs within 2 months after IFN withdrawal. Overall, the results suggest a poor long-term sensitivity of GBV-C to IFN, with a 0% rate of sustained virological response. It is of interest that one patient who did not respond to 3 mega units of IFN became GBV-C RNA-negative when the dose was increased to 9 mega units three times per week. Although this patient also experienced relapse after IFN withdrawal, it is possible that GBV-C could be sensitive to higher dosages or to different schedules of IFN alfa therapy (longer duration, daily administration). As discussed earlier, however, the effect of IFN on GBV-C replication did not appear to affect significantly the parameters of liver disease in these patients.

In summary, GBV-C infection is frequent in patients with chronic hepatitis C but does not seem to affect HCV replication, liver disease and responses of HCV infection and liver disease to IFN therapy. Whether GBV-C/HGV alone is able to induce acute or chronic liver disease remains to be demonstrated, but the relatively low prevalence of GBV-C/HGV in patients with non A to E hepatitis as determined by existing RT-PCR assays does not plead for a major role of this virus in liver diseases [Alter, 1996]. However, the possibility of more severe long-term consequences of GBV-C/HGV infection should not be precluded. In this context, it is highly probable that blood screening will have to be implemented with reliable GBV-C/HGV tests in the near future. Nevertheless, in the context of yet unidentified pathogenicity and of no effective available therapy, the question of patient information and medical decision in the case of detection of GBV-C/HGV infection remains unresolved.

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